on a water-insoluble carrier.

REMARKS

The Examiner maintains the rejection of the pending claims for the reasons set forth in the Final Office Action of August 27, 2002.

The Examiner indicated that the Toxicosis Study, vol. 2, p. 388, Fig. 2, 1989, submitted as Exhibit A in the response of November 27, 2002, was not a complete translation and as such, it was not considered. Applicants submit herewith Pathogenic Microorganism Detection Information, Vol 22, No. 8, page 3 (187), as Exhibit B. Applicants respectfully submit that all relevant parts of the reference (indicated in box) have been translated and that the attached represents a complete translation.

Claims 4 has been amended. Support for this amendment may be found for example, on page 1 lines 29-30 of the subject specification.

Rejection of Claims 4, 6 and 7 Under 35 U.S.C. §102(a)

The Examiner maintains the rejection of claims 4, 6 and 7 under 35 U.S.C. 102(a) as allegedly being anticipated by EP 0 993 834 A1 to Hirai et al. ("Hirai"). The Examiner asserts that Hirai's putative method of TSST-1 removal from body fluids by an adsorbent anticipates the claimed method for adsorptive removal of an enterotoxin in a body fluid. Applicants respectfully disagree.

The Examiner has stated that, "TSST-1 is not an enterotoxin"(Advisory Action of 1/23/03, p.3, line 14). However, regardless of the extensive documentation by the Applicants of unequivocal biochemical structural and functional differences between TSST-1 and the enterotoxins, the Examiner urges that because Hirai teaches removal of TSST-1 proteins, that "the skilled artisan could reasonably conclude that the method to remove enterotoxin from body fluids can be used to remove TSST-1 since enterotoxins and TSST-1 have similar characteristics".

The Examiner surmises that the invention "appears to be based on the molecular weight

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of the protein to be removed rather than the structure of the protein to be removed"; however, the Examiner does not identify what those "similar characteristics" could be and why one of skill would automatically apply a technique for removing TSST-1 from a bodily fluid to remove enterotoxins from a bodily fluid.

Finally, the Examiner continues to assert McLauchlin et al., (J. Food Prot. 2000 Apr; (63): 479-88), (Exhibit A; hereinafter "McLauchlin") as supporting the assertion that TSST-1 and enterotoxins are interchangable with respect to the subject claimed and prior art methods.

This response will reiterate the biochemical structural and functional differences between the TSST-1 and enterotoxins. It will also explain why one of ordinary skill in the art would not automatically apply a technique for removing TSST-1 from a bodily fluid to remove enterotoxins from a bodily fluid. Finally, this response will explain what McLauchlin actually means by its teaching of "96% agreement".

TSST-1 and enterotoxins diverge structurally and functionally. The attached article: Pathogenic Microorganism Detection Information, Vol 22, No. 8, page 3 (187) (Exhibit B), describes that enterotoxin F was renamed Toxic Shock Syndrome Toxin (TSST-1) because it was demonstrated to have no -S-S- bond within the molecule and to have a different structure from SE (staphylococcal enterotoxin). McLauchlin also teaches on page 479, right column, lines 10-15, that the staphylococcal exotoxin involved in toxic shock syndrome was initially designated as SEF but then later renamed TSST-1 and that TSST-1 has not been implicated in food poisoning and does not have the *in vivo* biological activities of true SEs. McLauchlin further teaches that TSST-1 and enterotoxins are not usually found in the same culture and therefore by extension, not often found in the same bodily fluid or patient. Table 2 shows only 35 out of 129 cultures examined (27%) contained both enterotoxins and TSST-1 as detected by differentially amplifying nucleic acids directing their expression. Additionally, Table 1 on page 481, lists the divergent nucleotide sequences of primers used for the detection of various SE and TSST-1 encoding genes. It is clear that the primers for SEA, SEB, SEC, SED, SEE, SEG, SEH, and SEI are quite different than those used to detect nucleic acids encoding TSST-1.

As properly recognized by the Examiner, one of skill in the art would not consider TSST-

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1 to be an enterotoxin. More importantly given this and past discussion of their structural and functional divergence, one of ordinary skill in the art would not expect to TSST-1 and enterotoxins to have sufficiently "similar characteristics", such that a method for removing TSST-1 from a body fluid would automatically lead them to presume that such a technique would also be effective in removing enterotoxins from a bodily fluid.

The only "similar characteristic" cited is size. Aside from the obvious biochemical structural and functional differences between the TSST-1 and enterotoxins, the Examiner maintains that because the method of Hirai "appears to be based on the molecular weight of the protein to be removed rather than the structure of the protein to be removed", the skilled artisan would reasonably conclude that the method to remove TSST-1 from body fluids can be used to remove enterotoxins.

Such argumentation overlooks the fact that the specification teaches that removal of enterotoxins from a bodily fluid is accomplished by contacting it with an enterotoxin adsorbent to remove the enterotoxin whereby the adsorbent comprises a compound with a particular log P. On page 5, line 32, the specification indicates that the log P value of a compound is a parameter of hydrophobicity. As such, the interaction of an enterotoxin adsorbent having a compound with a particular log P value with a protein in solution will be determined by its relative hydrophobicity (along with a number of other factors). This interaction is not, as the Examiner implies, determined solely on the basis of size of the protein to be adsorbed.

Even if one could assert that the method of Hirai "appears to be based on the molecular weight of the protein to be removed rather than the structure of the protein to be removed", nothing suggests to one of skill in the art to use the method of Hirai to remove enterotoxins from a bodily fluid. Table 1 of Exhibit B, shows that TSST-1 has a molecular weight of 22,049 Da as opposed to the larger molecular weights of enterotoxins: SEA (27,078 Da), SEB (28,386 Da), SEC1 (27,946 Da), SEC2 (27,589 Da), SEC3 (27,563 Da), SECD (26,360 Da), SEE (26,426) SEG (27,053 Da), SEH (25,145 Da), and SEI (24,928 Da). Under these circumstances, SEs are between 13.5% (SEI) and 28.7% (SEB) greater in mass than TSST-1 proteins. Moreover, the Table further contrasts the number of amino acids of each enterotoxin: SEA (233+24), SEB

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(239+27), SEC1 (239+27), SEC2 (239+27), SEC3 (239+27), SECD (288+30), SEE (230+27)
SEG (233+25), SEH (194+24), and SEI (217+24) with that of TSST-1 (194+40). As such, one

of skill would not assume that a particular method that is effective in removing TSST-1 based on

its size would be effective for removing enterotoxins based on their size.

Applicants respectfully assert that the has erroneously interpreted McLauchlin. The

Examiner opines that TSST-1 and enterotoxins are interchangable by stating that there is "96%

agreement between the PCR results for detecting Staphylococcal enterotoxin D and TSST-1"

(Advisory Action of 1/23/03, p. 3, line 16). McLauchlin **does not teach** that there is 96%

agreement, identity, homology, co-localization or any other similarity structural or functional,

between enterotoxin D and TSST-1. It simply teaches that detecting SED by PCR and reverse

latex agglutination assay gives you the same result 96% of the time. Ninety-six percent of the

time you will also get the same result when detecting TSST-1 by PCR and reverse latex

agglutination assay.

Moreover, the methods for detecting SEA-D and TSST-1 are not the same. Distinct toxin

gene-specific oligonculeotide primer pairs are used to distinguish between enterotoxin and

TSST-1 gene fragments and different commercial kits (See p. 480, left column, SET-RPLA,

Product Code TD940, Oxoid, for SEA-D and TSST-1-RPLA Product Code TD940, Oxoid, for

TSST-1) are used to distinguish between enterotoxin and TSST-1 protein contamination. As

such, the PCR used to detect enterotoxin D and TSST-1 encoding nucleotides is not the same,

nor is the reverse latex agglutination assays used to detect enterotoxin D and TSST-1 protein. A

comparison of the results for SEA-D and TSST-1 obtained from the production of toxins in vitro

as detected by RPLA (reverse passive latex agglutination assay) was made between the results of

amplification of the respective toxin gene fragments by PCR and described on page 482, left

column, line 15 to right column, line 3. It was found that there was complete agreement of 124

of 129 (96%) cultures, i.e. 96% agreement between the two detection methods. Accordingly,

there is nothing in McLauchlin to suggest any structural similarity between enterotoxin and

TSST-1 protein.

The Examiner alleges that the skilled artisan could reasonably conclude that the method

to remove enterotoxin from bodily fluids can be used to remove TSST-1 since enterotoxins and TSST-1, even though Hirai merely discloses that an adsorbent comprising a compound having a log P of at least 2.50 can remove and adsorb TSST-1. This line of reasoning implies that the Examiner considers the claimed invention unpatentably obvious. Regardless of the fact that the claims have not been rejected as such, obviousness requires a suggestion or motivation to modify the reference, and the prior art must suggest the desirability of the claimed invention. (MPEP 2143.01)

Hirai do not provide any suggestion or motivation to adsorb and remove any other compounds than TSST-1. There is no suggestion of the desirability or suitability of its method to remove enterotoxins from a bodily fluid. The references of record (McLauchlin, Schlievert and Mehrotra) merely disclose the facts that TSST-1 and enterotoxins are classified as superantigens and that PCR results and reverse passive latex agglutination assay have 96% agreement. There is no suggestion or motivation to apply Hirai's method to adsorb and remove the claimed enterotoxins. Additionally, a showing of obviousness requires that one of ordinary skill in the art have a reasonable expectation of success to arrive at the claimed invention. Applicant respectfully asserts that there is nothing Hirai that would provide the skilled artisan with any expectation of success. Hirai merely discloses that TSST-1 can be adsorbed and removed. Hirai does not suggest any compounds other than TSST-1 that could similarly be adsorbed and removed. Therefore, Hirai does not provide a reasonable expectation that its methods might work for removing enterotoxins from a bodily fluid.

Finally, the three references (McLauchlin, Schlievert and Mehrota) do not suggest that TSST-1 and enterotoxins have similar characteristic with regard to adsorption. Thus, they also do not provide any sufficient basis for a reasonable expectation for success.

The Examiner indicates that Applicants have not provided a "side-by-side" comparision to show that the method of the prior art differs from the claimed method. Applicant respectfully asserts that while the prior art teaches a method for removing TSST-1 from a body fluid, the claims recite a method for adsorptive removal of an enterotoxin in a body fluid. The Examiner has already concluded that TSST-1 is not an enterotoxin and can point to no teaching in the prior

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art that TSST-1 and enterotoxins have sufficiently "similar characteristics" such that one of skill in the art would deem them interchangeable in this context. As such, no further comparison is needed.

Accordingly, Hirai's putative method of TSST-1 removal from body fluids by an adsorbent neither anticipates nor renders unpatentably obvious the claimed method for adsorptive removal of an enterotoxin in a body fluid.

Applicants respectfully submit that claims 6 and 7, which depend from claim 4, are allowable for at least the same reasons as claim 4.
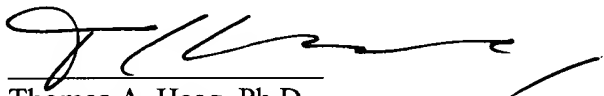
CONCLUSION

It is respectfully submitted that the present application is now in condition for allowance, which action is respectfully requested. The Examiner is invited to contact Applicants' representative to discuss any issue that would expedite allowance of the subject application.

It is not believed that any extensions of time or other fees are required in connection with the filing of this response. However, if any fees for extension(s) of time or additional fees are required in connection with the filing of this response, such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and the Commissioner is authorized to charge any such required fees or to credit any overpayment to Kenyon & Kenyon's Deposit Account No. 11-0600.

Respectfully submitted,

KENYON & KENYON

Dated: _May 27, 2003_

By: Thomas A. Haag, Ph.D.
(Reg. No. 47,621)

1500 K Street, N.W.
Washington, D.C. 20005
(202) 220-4200
(202) 220-4322 (direct)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS:

Please amend claim 4 as follows:

4. (Thrice Amended) A method for adsorptive removal of an enterotoxin in a body fluid

which comprises contacting an enterotoxin-containing body fluid with an

enterotoxin adsorbent to adsorb and remove the enterotoxin, wherein said enterotoxin is

at least one selected from the group consisting of staphylococcal enterotoxins A, B, C1,

C2, C3, D, E, G, H and I,

said adsorbent comprising a compound with a log P, in which P respresents a

partition coefficient in an octanol-water system, value of not less than 2.50 as

immobilized on a water-insoluble carrier.

EXHIBIT A

The Detection of Enterotoxins and Toxic Shock Syndrome Toxin Genes in *Staphylococcus aureus* by Polymerase Chain Reaction

J. McLAUCHLIN,[1]* G. L. NARAYANAN,[1] V. MITHANI[1], AND G. O'NEILL[2]

[1]*Food Safety Microbiology Laboratory, PHLS Division of Gastrointestinal Infections, and* [2]*Laboratory of Hospital Infection, Central Public Health Laboratory, 61 Colindale Ave., London NW9 5HT, UK*

ABSTRACT

A simple polymerase chain reaction (PCR)-based procedure was developed for the detection of fragments of staphylococcal enterotoxins (SEs) SEA, SEB, SEC, SED, SEE, SEG, SEH, and SEI together with the toxic shock syndrome toxin (TSST-1) genes of *Staphylococcus aureus*. One hundred and twenty-nine cultures of *S. aureus* were selected, 39 of which were recovered from 38 suspected staphylococcal food-poisoning incidents. The method was reproducible, and 32 different toxin genotypes were recognized. The presence of SE genes was associated with *S. aureus* strains reacting with phages in group III, and the TSST-1 gene with phages in group I. There was a 96% agreement between the PCR results for detection of SEA–D and TSST-1 as compared with a commercial reverse passive latex agglutination assay for the detection of SEs from cultures grown in vitro. Enterotoxin gene fragments were detected in *S. aureus* cultures recovered from 32 of the 38 suspected staphylococcal food poisoning incidents, and of these, 17 were associated with SEE, SEG, SEH, and SEI in the absence of SEA–D. Simple PCR procedures were also developed for the detection of SE directly in spiked food samples, and this was most successfully achieved in mushroom soup and ham. Detection was less successful in three types of cheese and in cream. SEA or SEB were detected by enzyme-linked immunosorbent assay in three food samples (two of which were associated with food poisoning incidents) naturally heavily contaminated with *S. aureus*: the appropriate SEA or SEB gene fragments were detected directly in these three foods by PCR.

Staphylococcal food poisoning (SFP) is an intoxication resulting from the ingestion of food or beverages containing preformed enterotoxins (SEs) usually produced by *Staphylococcus aureus (17)*. SFP is characterized by the sudden onset of nausea, vomiting, abdominal cramps, and diarrhea within 2 to 6 h of ingestion of toxin and generally lasts from <12 h to 2 days. Recovery is usually within 1 to 3 days; however, hospitalization may be necessary *(17)*. Although of declining incidence in the United Kingdom *(20)*, SFP remains a reported cause of food poisoning, and control of this disease is of considerable economic importance to food industries worldwide *(6, 17)*. The diagnosis of SFP is based primarily on the symptoms presented by the patient together with the recovery of enterotoxigenic staphylococci for foods or food handlers and/or the detection of SEs in food remnants *(6, 17)*.

SEs are single-chain proteins of molecular weight 26,900 to 29,699 Da, and 11 different SEs have been described: i.e., SEA, SEB, SEC1, SEC2, SEC3, SED, SEE, SEG, SEH, SEI, and SEJ *(11, 12, 17, 23)*. In the United Kingdom, SEA alone, SEA plus SED, and SEC plus SED are responsible for 57%, 15%, and 8%, respectively of the staphylococcal food poisoning incidents that were reported between 1969 and 1990, although the previous scope of SE testing was limited to SEA, SEB, SEC, SED, and SEE only *(20)*.

SEs can be detected by their biological activity in vivo or in vitro. However, for practical purposes, routine detection by immunoassays (i.e., immunodiffusion, radioimmunoassay, latex agglutination, immunoblotting, or enzyme-linked immunosorbent assay [ELISA]) are almost universally used *(17)*. Because DNA sequence information is available for all described SEs *(11, 12, 16, 17)*, PCR now offers an alternative method for the detection of these toxin genes *(2, 7)*.

A further staphylococcal exotoxin involved with toxic shock syndrome has been described *(3)*. This toxin was initially designated as SEF but was later renamed as the toxic shock syndrome toxin-1 (TSST-1) *(3)*. TSST-1 has not been implicated in food poisoning and does not have the in vivo biological activities of the true SEs *(17)*. However, because the actions of SEs are involved with some nonfoodborne human diseases similar to toxic shock syndrome *(9)*, there is a need for clinical microbiologists to offer diagnostic tests for the detection of both TSST-1 and SEs.

The Public Health Laboratory Service Food Safety Microbiology Laboratory (FSML) has previously been active in the development and evaluation of methods for the detection of SEs and TSST-1 *(15, 18–20)* and currently uses commercially produced reverse passive latex agglutination (RPLA) assays in its reference toxin testing service *(18)*. These assays are able to detect SEA, SEB, SEC, SED, and TSST-1 only. There is evidence, however, that SEs other than SEA–D are involved with food poisoning in the United Kingdom *(10, 20)*.

* Author for correspondence. Tel: + 44 020 8200 4400; Fax: + 44 020 8200 8264; E-mail: jmclauchlin@phls.nhs.uk.

The purposes of this present study were to develop PCR-based procedures for the detection of SEs and TSST-1 and to assess (i) the presence of different SEs in the cultures of S. aureus submitted to the FHL (including cases of suspected SFP), particularly those where SEs were not detected using the RPLA procedure; (ii) their use in epidemiological typing of S. aureus isolates; and (iii) their application to the direct detection of SE gene fragments in foods that were both artificially and naturally contaminated by S. aureus.

MATERIALS AND METHODS

Cultures of staphylococci. Cultures of S. aureus were selected from isolates submitted to the FHL and were stored on nutrient agar slopes at room temperature in the dark. Also included were two cultures that had previously been shown to produce SEG and SEH in vitro (donated by ACL Wong, Food Research Institute, Madison, Wis.). Cultures were confirmed as S. aureus by colonial appearance on horse-blood agar, coagulase, and thermostable nuclease production as described elsewhere (1, 8). Isolates were cultured using Columbia horse-blood agar (Oxoid, Basingstoke, UK) at 37°C throughout. Staphylococcus epidermidis (NCTC 11047) was used as a nonexotoxigenic control throughout and was cultured as described above. All S. aureus isolates were further characterized by phage typing in the PHLS Staphylococcus Reference Section (PHLS Laboratory of Hospital Infection, Central Public Health Laboratory, London, UK) using the International Basic Set of 23 phages as described elsewhere (5). Isolation histories, food types, and brief epidemiological information concerning incidents of food poisoning were collected for each culture.

Detection of SEs and TSST-1 produced by cultures of S. aureus grown in vitro. RPLA assays for SEA–D and TSST-1 were performed using the SET–RPLA (product code TD900, Oxoid) and TSST-1–RPLA (product code TD940, Oxoid) test kits, respectively. Cultures were tested as described previously (18).

An ELISA test (Bommeli, Berne, Switzerland) was also used for the detection of SEA, SEB, SEC, and SED in both culture supernatants and in food extracts as described previously (19).

Detection of enterotoxin genes by PCR: Target DNA extraction. Purified DNA for use as positive and negative control material in each batch of PCR reactions was prepared by harvesting bacterial cells grown overnight, shaking the cells in the presence of guanidinium thiocyanate (4) and 0.1-mm-diameter zirconium beads in a Beadbeater-8 (Stratech Scientific, Luton, UK) at maximum speed for 30 s, followed by DNA extraction using activated silica (4). The purified DNA samples were stored at −40°C. Negative control DNA was prepared from S. epidermidis and positive controls from S. aureus previously identified as containing the appropriate exotoxins.

For detection of enterotoxin genes in wild-type S. aureus cultures, five to six colonies were harvested from blood agar, suspended in 200 μl of nuclease-free water (product code W4502, Sigma-Aldrich, Poole, UK), vortexed for approximately 5 s, and centrifuged at $10,000 \times g$ for 1 min. The supernatant containing target DNA was removed and either used immediately or stored on ice and used within 30 min.

PCR. Primers used for the detection of SEA–E and TSST-1 were as described by Johnson and colleagues (7). DNA sequences are available for SEG, SEH, and SEI (11, 12, 16), and primers were designed from EMBL sequences AF064773, U11702, and AF064774 for SEG, SEH, and SEI, respectively, using the Genetics Computer Group Wisconsin package, version 9.1, Madison, Wis. The programs used were "prime" for primer design, "pileup" for multiple sequence alignment, and "find patterns" to confirm that the primers were designed for the correct genes. The sequences of all primers together with their respective amplified fragments are shown in Table 1.

PCR amplification was performed in 25-μl total volumes and included 2 μl of target DNA. Positive controls using purified DNA for the appropriate exotoxin and two negative controls (S. epidermidis DNA and buffer only) were included in each batch of tests. The following concentrations of the various reagents were used: 1× PCR buffer (Gibco Life Technologies, Paisley, UK); 5 mM MgCl₂; 0.2 mM dNTP mix (Gibco Life Technologies); 1 μM of each primer pair; 0.05% (vol/vol) W1 (Gibco Life Technologies); and 1 unit Taq polymerase (Gibco Life Technologies). A single PCR protocol was used throughout and was performed in a Uno II thermocycler (Biometra, Göttingen, Germany) and comprised of: 94°C for 4 min; 30 cycles of 94°C for 2 min, 55°C for 2 min, and 72°C for 1 min; followed by a final extension at 72°C for 7 min.

A 5-μl aliquot of the PCR products was examined following electrophoresis in 1.6% agarose/ethidium bromide gels. All gels were recorded using UV transillumination and Type 52 film (Polaroid Ltd., St. Albans Herts, UK).

Detection of SEs and gene sequences in contaminated samples of foods. Foods (canned mushroom soup, full fat cream, cottage cheese, mozzarella cheese, full fat hard cheese, and sliced ham) representative of potential vehicles for SFP were purchased from retail outlets. All foods were tested to ensure an initial absence of S. aureus as described elsewhere (13). Thermonuclease activity of the foods was detected using metachromic agar (8), and numbers of S. aureus present in the samples were estimated using a surface spread or surface drop enumeration method (13).

Foods were spiked with cultures of previously characterized exotoxigenic S. aureus by evenly distributing 100 μl of a suspended culture to either 0.5 ml of liquid food or 0.5 g of solid or semisolid food. Two series of spiking experiments were performed. The first was used to investigate the sensitivity of the method by inoculating different levels of S. aureus onto foods and the second to simulate temperature-abused contaminated foods where growth of S. aureus had occurred. In the first series of experiments, 0.5 g/ml of food samples were inoculated with 10^7, 10^6, 10^5, or 10^4 CFU of S. aureus (previously shown to contain the SEA gene) and homogenized in 5 ml of water. A control was included where each dilution of S. aureus was directly homogenized into 5 ml of nuclease-free water. The aqueous extracts were then decimally diluted and used as target DNA for PCR reactions; a negative control was included comprising a heterologous primer pair. Because each PCR reaction used 2 μl of the total 5-ml aqueous food extract, this represents a 1/2,500 dilution, and an estimate of the maximum numbers of S. aureus in each reaction was calculated on the basis of this dilution. The second series of experiments involved inoculation of foods with approximately 10^6 CFU of S. aureus cultures, followed by incubation for 18 h at 37°C. Levels of S. aureus within 30 min after inoculation and after incubation were estimated, and aqueous extracts, dilutions, and PCR procedures were performed as above.

Food samples (pasta, corned beef, and cake) were also obtained that had been submitted to the FSML as part of followup investigations of food poisoning or contamination incidents and were naturally contaminated with S. aureus. These foods were extracted as described elsewhere in this section.

TABLE 1. *Sequence of primers for the detection of staphylococcal enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1) gene fragments[a]*

Primer designation	Nucleotide sequence, 5' to 3'	Target enterotoxin gene	Fragment size (base pairs)	Reference
SEA1	TTG GAA ACG GTT AAA ACG AA	SEA	120	7
SEA2	GAA CCT TCC CAT CAA AAA CA			
SEB1	TCG CAT CAA ACT GAC AAA CG	SEB	478	7
SEB2	GCA GGT ACT CTA TAA GTG CC			
SEC1	GAC ATA AAA GCT AGG AAT TT	SEC	257	7
SEC2	AAA TCG GAT TAA CAT TAT CC			
SED1	CTA GTT TGG TAA TAT CTC CT	SED	317	7
SED2	TAA TGC TAT ATC TTA TAG GG			
SEE1	TAG ATA AAG TTA AAA CAA GC	SEE	170	7
SEE2	TAA CTT ACC GTG GAC CCT TC			
TSST1	ATG GCA GCA TCA GCT TGA TA	TSST-1	350	7
TSST2	TTT CCA ATA ACC ACC CGT TT			
SEG1	TGC TAT CGA CAC ACT ACA ACC	SEG	704	This study
SEG2	CCA GAT TCA AAT GCA GAA CC			
SEH1	CGA AAG CAG AAG ATT TAC ACG	SEH	495	This study
SEH2	GAC CTT TAC TTA TTT CGC TGT C			
SEI1	GAC AAC AAA ACT GTC GAA ACT G	SEI	630	This study
SEI2	CCA TAT TCT TTG CCT TTA CCA G			

[a] The base positions for the primers developed in this study were as follows: SEG1 bases 61–81, and SEG2 bases 745–764 from EMBL AF064773; SEH1 bases 272–292 and SEH2 bases 745–766 from EMBL U11702; SEI1 bases 51–72, and SEI2 bases 659–680 from EMBL AF064774.

To prepare target DNA for PCR procedures from dry (pasta), semisolid (corned beef, cake, cream, cottage cheese), and solid (hard cheese, mozzarella cheese, ham) foods, 0.5 g of the artificially contaminated food was suspended in 5 ml of nuclease-free water and periodically vortex-mixed for up to 60 min until evenly suspended. For the liquid food (soup), the 0.5-ml sample was similarly suspended in 5 ml of nuclease-free water and vortex-mixed for approximately 30 s. The resulting homogenates were centrifuged at 10,000 × *g* and the supernatants decimally diluted 1:10, 1:100, and 1:1,000 in nuclease-free water. Two microliters of the neat suspension and each of the dilutions were used as target DNA in PCR reactions as described above.

Following PCR procedures, the resulting amplicons were detected by agarose/ethidium bromide electrophoresis and the banding intensity was semiqualitatively recorded as ± (faint), + (moderate), or ++ (strong).

RESULTS

One hundred twenty-nine cultures of *S. aureus* were selected and these included 38 isolates from foods suspected to be associated with food poisoning. One further culture was included from a patient's fecal specimen but differed from the incriminated food isolate (see below): these are considered as two different strains. The remaining 90 isolates were from foods (85 cultures), patient specimens not associated with SFP (3 cultures), or comprised 2 cultures previously shown to produce SEG or SEH. A single culture was selected for each contamination or food poisoning incidents unless phage-typing results suggested the presence of multiple strains (see below).

Results of testing the total 129 cultures of *S. aureus* by PCR for the presence of SEs and TSST-1 are shown in Table 2. Overall, in 42 *S. aureus* cultures (32.5%) only one

exotoxin gene was detected by PCR: more than one exotoxin gene was detected in 63 cultures (49%), and 24 (19%) contained three or four different exotoxin genes. No exotoxin genes were detected in 24 (18.5%) of the cultures. Gene fragments from SEE, SEG, SEH, or SEI were detected in 34 cultures (26%) in which SEA, SEB and SEC, and SED were not detected either by PCR or RPLA (see below) and had therefore not previously been identified as enterotoxigenic. PCR identified SEG and SEH gene fragments in the two cultures shown previously to contain these genes.

The associations between the SE and TSST-1 gene fragments and the phage groups are shown in Table 2. Among the 24 cultures where no exotoxin genes were detected, 16 (67%) were either nonphage-typable or reacted with phages in groups other than I and III. In contrast, of the 105 cultures where exotoxin genes were detected, 38 (36%) were similarly either nonphage-typable or reacted with phages in groups other than I and III. Overall, 46 (45%) out of the 102 cultures where SEs were detected reacted with phages within group III. Similarly, 18 out of the 34 cultures (53%) where SEA–D were not detected but where SEE, SEG, SEH, or SEI genes were present also reacted with phages within group III. Among the 38 cultures where the TSST-1 gene fragment was detected, 20 (53%) were associated with phage-group I.

Altogether 32 different toxin genotypes were recognized (Table 2). To assess the stability of these genotype results, 41 cultures were tested on two or more occasions, and identical SE types were obtained with each culture. A further 10 isolates were selected from five contamination

TABLE 2. *Summary of results for detection of SEs and TSST-1 gene fragments by PCR and phage group associated with 129 cultures of S. aureus*

SE gene fragments detected by PCR	Total numbers of cultures (numbers from suspected food poisoning incidents)	Phage groups					
		I	I/III	II	III	Other	NT[a]
SEA	10 (2)		3		5	1	1
SEA/SEB	1 (1)		1				
SEA/SEC/TSST-1	2						2
SEA/SED	3 (1)		1		2		
SEA/SEG/SEH	1						1
SEA/SEH	2 (2)		1			1	
SEA/TSST-1/SEG	8 (2)	4	1		1	1	1
SEA/TSST-1/SEG/SEI	1 (1)	1					
SEB	4 (1)						4
SEB/SEC/SEG/SEI	1 (1)					1	
SEB/SEG	1 (1)		1				
SEB/SEG/SEI	4 (1)				1	2	1
SEB/SEH	1				1		
SEC	7	3	1	1	2		
SEC/SED/SEG/SEI	1 (1)						1
SEC/SEG	2						2
SEC/SEG/SEI	1					1	
SEC/SEH	1						1
SEC/TSST-1	15 (1)	4	3	1	4	1	2
SEC/TSST-1/SEE	1					1	
SEC/TSST-1/SEG	1						1
SEE	1						1
SEE/TSST-1	1 (1)						1
SEG	8 (6)	1	1	2	3	1	
SEG/SEH/SEI	1 (1)						1
SEG/SEI	8 (3)	1	2		3	1	1
SEH	8 (5)		1		7		
SEI	1 (1)		1				
TSST-1/SEG/SEI	1						1
TSST-1	3 (1)	1	1				1
TSST-1/SEG	4	4					
TSST-1/SEG/SEI	1	1					
ND[b]	24 (6)		3	6	5	4	6
Totals	129 (39)	20	21	10	34	15	29

[a] NT, not typable.
[b] ND, not detected.

or suspected SFP incidents where between two and five cultures were recovered (i.e., different colonies selected from the same primary isolation plate, food, and related patient isolates, or as a result of the examination of different foods from the same manufacturer or origin). Results of phage-typing together with exotoxin detection by RPLA and SE gene detection by PCR are shown in Table 3. Analysis of isolates from incidents 1, 3, 4, and 5 by PCR and phage-typing (as well as by RPLA) were consistent with the same strain having been recovered on multiple occasions (Table 3). Evidence for a different strain isolated from two foods within incident 2 and between the patient and food strain in incident 3 was obtained by all methods (Table 3).

A comparison of the results for SEA–D and TSST-1 obtained from the production of toxins in vitro (as detected by RPLA) was made between the results of amplification of the respective toxin gene fragments by PCR. There was complete agreement for 124 (96%) of the 129 cultures: concordant positive results were obtained by both methods from 75 cultures, and SEA–D and TSST-1 were not detected in 49 isolates. Details of the five discrepant results are given in Table 4. Four of the five cultures where the results from the two methods were inconsistent were further tested by ELISA for the presence of SEA, SEB, and SEC (Table 4). Culture number 3 (Table 4) that was RPLA positive for TSST-1 but negative by PCR was not tested by ELISA because the commercial kit is not designed to detect TSST-1. Results were consistent between the RPLA and ELISA with three of the isolates (culture numbers 1, 2, and 5) and were consistent between the ELISA and PCR in one isolate (culture 4).

TABLE 3. *Analysis of multiple isolates of* S. aureus *recovered from five incidents of food contamination or suspected staphylococcal food poisoning*

Incident[a]	Sources of isolates	RPLA[b]	PCR[c]	Individual phage reactions
1	Rice	ND[d]	ND	3A
	Rice	ND	ND	3A
2	Cooked beef	SEB, SEC	SEB, SEC, SEG, SEI	95
	Cooked ham	ND	SEH	29, 52A, 79, 6, 42E, 47, 53, 54, 77, 83a, 85
3	Sweet and sour sauce	ND	SEG, SEI	29, 94, 96
	Battered pork	ND	SEG, SEI	29, 94, 96
	Boiled rice	ND	SEG, SEI	29, 94, 96
	Feces	ND	ND	3A, 3C, 55, 71
4	Ras malai	ND	SEG	29, 52, 52A, 79, 80, 95, 47, 53, 54, 77, 83A, 84, 85
	Ras malai	ND	SEG	29, 52, 52A, 79, 80, 95, 47, 53, 54, 77, 83A, 84, 85
5	Cheese	SEA, TSST-1	SEC, TSST-1	75, 77, 83A, 84
	Cheese	SEA, TSST-1	SEC, TSST-1	75, 77, 83A, 84
	Cheese	SEA, TSST-1	SEC, TSST-1	75, 77, 83A, 84
	Cheese	SEA, TSST-1	SEC, TSST-1	75, 77, 83A, 84
	Cheese	SEA, TSST-1	SEC, TSST-1	75, 77, 83A, 84

[a] Incidents 3 and 4 involved cases of suspected staphylococcal food poisoning.
[b] Tested by RPLA for SEA, SEB, SEC, SED, and TSST-1.
[c] Tested by PCR for gene fragments of SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and TSST-1.
[d] ND, not detected.

Enterotoxin gene fragments were detected in *S. aureus* cultures recovered from 32 of the 38 suspected SFP incidents (Table 2). Of the 38 suspected incidents, 9, 4, and 2 were due to SEA, SEB, or SEC (alone or with another enterotoxin), respectively. Among the cultures from the nine possible SFP incidents associated with SEA, other enterotoxin genes were detected in seven of the cultures: one with SEB, one with SED, two with SEH, two with SEG, and one with SEG and SEI (Table 2). Of the remaining 23 incidents not associated with SEA–D (i.e., cultures previously identified as nonenterotoxigenic), *S. aureus* containing SEE, SEG, SEH, or SEI were detected in 17 (45%). Overall, SEA, SEG, SEH, and SEI were most commonly

detected and were found (together with other toxins) in 9 (23%), 17 (44%), 8 (21%), and 9 (21%) of the incidents, respectively: SEG alone was associated with 6 (16%) of the incidents. Enterotoxin gene fragments were not detected in cultures collected from the final six incidents, although the TSST-1 gene and toxin was detected by both PCR and RPLA from one culture. A brief summary of the suspected SFP incidents where no enterotoxin genes or only SEE, SEG, SEH, and SEI were detected is shown in Table 5.

To investigate further applications of the PCR procedures developed for analysis of cultures, the PCR techniques were directly applied to extracts from foods. Two series of experiments were designed using foods inoculated with *S. aureus*, and this bacterium was not recovered from any of the uninoculated foods, and extracts from these did not amplify SE gene fragments. Results of detection of SEA by PCR in foods inoculated with different levels of *S. aureus* are shown in Table 6. Some inhibition of the PCR reactions from the food extracts was apparent, but this could be partially overcome by dilution (see also Tables 6 and 7). Examination of the control sample (viable cells diluted in water), indicated that the limit of sensitivity for detection of the SEA gene was ≤4 viable *S. aureus* per test. A similar (albeit slightly lower) level of sensitivity of 4 to 40 CFU of *S. aureus* was achieved in the artificially contaminated ham and soup. However, detection of SE genes was poor or was not achieved in the four dairy products (cheese and cream; Tables 6 and 7). Primer pairs heterologous to the inoculated *S. aureus* were included for

TABLE 4. *Results of detection of SE and TSST-1 genes by RPLA, PCR and ELISA in five cultures of* S. aureus *giving discrepant results*

Culture number	Results of testing by:		
	RPLA[a]	PCR[a]	ELISA[b]
1	SEB	ND	SEB
2	ND[c]	SEC	ND
3	SEC, TSST-1	SEC	Not tested
4	SED	ND	ND
5	SEA, TSST-1	SEC, TSST-1	SEA

[a] Results of testing for SEA, SEB, SEC, SED, and TSST-1.
[b] Results of testing for SEA, SEB, and SEC.
[c] ND, not detected.

TABLE 5. *Possible outbreaks of staphylococcal food poisoning associated with SEs other than SEA-D[a]*

Number ill/ at risk[a]	Incubation period/symptoms	Food type	S. aureus CFU/g of food	Results of SE detection from cultures of S. aureus grown in vitro by:	
				RPLA[b]	PCR[c]
1/NK	2.5–5 h/D, V	Corned beef	5×10^4	TSST-1	SEE/TSST-1
9/10	NK/V, D	Reheated chicken	NK	ND	SEG
18/NK	3.5–5.5 h/V, D	Cooked chicken	NK	ND	SEG
2/2	8 h/NK	Crab meat	8×10^8	ND	SEG
10/80	NK/NK	Gammon	10^5	ND	SEG
2/2	6–24 h/SC, D	Ras-malai takeaway	1.4×10^3	ND	SEG
1/NK	3–6 h/V, AP	Unpasteurized cheese	NK	ND	SEG
43/45	3 h/NK	Prawns	5×10^5	ND	SEH
12/100	2.5–4 h/V, D	Corned beef	3×10^5	ND	SEH
14/25	NK/AP	Cooked beef and ham	NK	ND	SEH
2/NK	4 h/N, V, D	Ham	10^3	ND	SEH
3/NK	NK	Corned beef	NK	ND	SEH
4/NK	NK/D, SC	Cooked turkey	1.26×10^5	ND	SEI
1/NK	2–3 h/D, V	Prawns	4.9×10^4	ND	SEG, SEI
3/20	NK/NK	Sausages	NK	ND	SEG, SEI
6/NK	NK/V	Chinese meal	NK	ND	SEG, SEI
3/NK	2–3 h/V, D	Corned beef	1×10^5	ND	SEG, SEH, SEI
21/56	NK/NK	Chicken	NK	ND	ND
3/3	4 h/V	Egg sandwich	1.5×10^7	ND	ND
10/NK	2.5–5 h/D, V	Corned beef	5×10^4	TSST-1	TSST-1
1/2	1 h/V	Take-away meal	6.0×10^2	ND	ND
1/NK	2 h/SC, N, V	Egg fried rice	NK	ND	ND
NK/NK	7 h/V, D	Fried rice	5×10^5	ND	ND

[a] NK, not known; V, vomiting; SC, stomach cramps; D, diarrhea; AP, abdominal pain; N, nausea.
[b] Tested by RPLA for SEA, SEB, SEC, SED, and TSST-1.
[c] Tested by PCR for gene fragments of SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and TSST-1.

each food, and no PCR amplification was detected (results not given).

Results of testing artificially contaminated and temperature-abused foods are shown in Table 7. Growth of the inoculated organisms was detected in the soup, cream, and ham; some survival was detected in the mozzarella cheese and hard cheese; no viable *S. aureus* were recovered from the cottage cheese. A range of different *S. aureus* strains was used in the inoculation of the soup, and amplification of SE fragments from all was successfully achieved, albeit some of the toxin genes were more readily detected (Table 7). In the three foods where growth of *S. aureus* had been detected (soup, cream, and ham) the appropriate SE gene fragments were detected, although this was least successfully achieved in the cream sample. SEA and SED fragments were detected in the hard cheese and SEA in the mozzarella, although only moderate amplification was achieved. No amplification of the SED gene was detected with the contaminated cottage or mozzarella cheese samples or of the SEA gene in the cottage cheese. Primer pairs heterologous to the inoculated *S. aureus* were included for each food, and no PCR amplification was detected (results not given).

To evaluate further the PCR technique, three food samples were obtained that had been submitted to the FSML as part of followup investigations and were naturally contaminated with *S. aureus*. Two of the samples (pasta and

corned beef) were associated with SFP. The pasta (dried lasagne) identified 15 years previously as contaminated with *S. aureus* producing SEA was responsible for a large international outbreak of SFP (22) and had subsequently been stored dry and at room temperature. The sample of cake was not identified as associated with illness. Results of testing the three foods naturally contaminated with *S. aureus* by PCR are shown in Table 8. All foods had been heavily contaminated ($>10^7$ CFU/g) with *S. aureus*, although at the time of PCR testing, the bacteria in the pasta were no longer viable. SEA (pasta and corned beef) and SEB (cake) had been detected by ELISA in the foods and were also detected from the appropriate cultures grown in vitro by RPLA. The appropriate SEA and SEB gene fragments were detected by PCR in the food samples; an SEG gene fragment was also detected in the corned beef.

DISCUSSION

There is a need for fast, reliable, nonsubjective, cost-effective, specific, and sensitive assays for the detection of a range of bacterial toxins. These assays may rely on the detection of biological activity (either by infected or exposed experimental animals or using in vitro assays) or on immunoassays that usually require purification of the toxins for production of specific antibodies. An alternative to these two approaches is the detection of specific toxin gene sequences by PCR. Detection of specific enterotoxin gene

TABLE 6. *Results of analysis of foods inoculated with different levels of* S. aureus *(SEA producer) and tested for the SEA gene fragment by PCR*

Food type	CFU of S. aureus/g or ml inoculated onto food	PCR test results, dilutions of food sample[a]			
		Neat	1:10	1:100	1:1,000
Estimated maximum number of S. aureus in each reaction		4,000	400	40	4
Water (positive control)		++	++	+	±
Mushroom soup		++	+	+	−
Cottage cheese		−	+	+	−
Mozzarella cheese	10^7	−	−	−	−
Hard cheese		−	−	−	−
Ham		+	+	++	±
Cream		NT[a]	NT	NT	NT
Estimated maximum number of S. aureus in each reaction		400	40	4	0.4
Water (positive control)		++	+	±	−
Mushroom soup		−	+	+	−
Cottage cheese		+	+	−	−
Mozzarella cheese	10^6	−	−	−	−
Hard cheese		−	−	−	−
Ham		++	+	+	+
Cream		±	−	−	−
Estimated maximum number of S. aureus in each reaction		40	4	0.4	0.04
Water (positive control)		+	±	−	−
Mushroom soup		−	−	−	−
Cottage cheese		−	−	−	−
Mozzarella cheese	10^5	−	−	−	−
Hard cheese		−	−	−	−
Ham		+	−	−	−
Cream		±	−	−	−
Estimated maximum number of S. aureus in each reaction		4	0.4	0.04	0.004
Water (positive control)		±	−	−	−
Mushroom soup		−	−	−	−
Cottage cheese		−	−	−	−
Mozzarella cheese	10^4	−	−	−	−
Hard cheese		−	−	−	−
Ham		−	−	−	−
Cream		−	−	−	−

[a] Banding intensity was recorded as strong (++), moderate (+), or weak (±). NT = not tested; − = not detected.

fragments, however, does not necessarily indicate the ability of an organism to produce intact and biologically active toxin or to produce sufficient toxin to induce disease. Hence, while detection of a toxin gene by PCR may indicate the potential to cause disease, the detection of biologically active toxin should be regarded as the gold standard. As previously described, *S. aureus* produces at least 11 different enterotoxins and, although it is possible to detect the biological activities of these toxins in animals *(17)*, it is not practicable (or ethical) to perform these as routine assays. In vitro assays to detect the biological activity of these toxins are not well developed, and immunoassays are only available for a limited range of the SEs *(17)*. Because DNA sequences are available for all the SEs so far described,

PCR offers great potential for the investigation of SFP (albeit with the shortcomings described above), especially in elucidating the role of the more recently described enterotoxins.

A simple and rapid method for the detection of gene fragments from eight different SEs and the TSST-1 in cultures of *S. aureus* is reported here. The method is reproducible, nonsubjective, and offers the added benefit of a discriminatory genetic tool for epidemiological typing. The PCR method was reliable in that it showed a high degree of agreement with the results obtained for SEA–D and TSST-1 using the RPLA kit, and correctly identified SEG and SEH gene sequences in cultures obtained from elsewhere. The cost of materials to test one culture for all nine

TABLE 7. *Results of analysis of foods inoculated with* S. aureus *incubated overnight at 37°C and tested for the presence of SEs and TSST-1 gene fragments by PCR[a]*

| Food type | Exotoxin type of S. aureus inoculated on to food | S. aureus CFU/g or ml | | PCR test results, dilution of food | | | |
		Within 30 min of inoculation	After incubation overnight at 37°C	Neat	1:10	1:100	1:1,000
Tinned mushroom soup	A	9×10^4	8×10^8	−	−	+ +	+
	B	5×10^4	2×10^9	−	−	+ +	−
	C	1×10^5	2×10^9	−	+	+ +	+
	D	2×10^5	2×10^9	−	−	+	−
	E	3×10^5	1×10^9	+ +	+ +	+ +	+
	TSST-1	4×10^4	1×10^9	−	−	+	+
	G	1×10^6	3×10^9	−	−	+ +	+ +
	H	4×10^6	1×10^9	−	+ +	+ +	+ +
	I	2×10^6	3×10^9	−	−	+	−
	None	ND	ND	−	−	−	−
Cream	A	4×10^6	1×10^8	−	+ +	+	−
	D	2×10^7	5×10^7	−	+	−	−
	None	ND	ND	−	−	−	−
Cottage cheese	A	4×10^6	ND	−	−	−	−
	D	2×10^7	ND	−	−	−	−
	None	ND	ND	−	−	−	−
Mozzarella cheese	A	4×10^6	7×10^6	+	−	−	−
	D	2×10^7	7×10^5	−	−	−	−
	None	ND	ND	−	−	−	−
Hard cheese	A	4×10^6	ND	+	−	−	−
	D	2×10^7	4×10^5	+	+	−	−
	None	ND	ND	−	−	−	−
Ham	A	4×10^6	6×10^8	+ +	+ +	+	±
	D	2×10^7	7×10^8	+ +	+ +	+	−
	None	ND	ND	−	−	−	−

[a] All samples were tested by PCR with at least one set of primers heterologous to the exotoxin inoculated into the food sample and amplicons were not detected. ND = not detected.

TABLE 8. *Results of detection of SEs by PCR directly in foods naturally contaminated with* S. aureus

| Food type | CFU of S. aureus/g of food sample | Thermo-nuclease test on food sample | SE detection in food sample[a] | SE produced by S. aureus grown in vitro[b] | PCR test results, dilutions of food sample | | | |
					Neat	1:10	1:100	1:1,000
Pasta	Not detected[d]	−	SEA	ESA				
PCR test for SEA					+ +	+ +	+	+
PCR test for all other primer pairs[c]					−	−	−	−
Cake	2×10^7	+	SEB	SEB				
PCR test for SEB					+ +	+ +	+ +	+ +
PCR test for all other primer pairs[c]					−	−	−	−
Corned beef	4×10^7	+	SEA	SEA and TSST-1				
PCR test for SEA					+	+ +	+	+
PCR test for TSST-1					−	+ +	+	−
PCR test for SEG					−	+ +	+	−
PCR test for all other primer pairs[c]					−	−	−	−

[a] Tested for SEA, SEB, SEC, and SED only by ELISA.
[b] Tested for SEA, SEB, SEC, SED, and TSST-1 only by RPLA.
[c] All samples were tested by PCR for gene fragments of SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and TSST-1.
[d] Sample of lasagna involved with an outbreak in 1983, previously shown to contain 2×10^8 CFU S. aureus/g that produced SEA (22).

genes by PCR is estimated as approximately £11.50 (US$18.50). This cost is comparable to both the estimate for the RPLA tests (£8.00 or US$13) that is currently routinely used in this laboratory and that detects only five exotoxins, and to the costs of between US$8 and 13.50 (£5 and 8.50) previously estimated for other commercial tests (16). The PCR and RPLA procedures require comparable labor time to perform, but the turnaround time for the PCR test is 1 or more days quicker than that for the RPLA test. Improvements in PCR procedures, such as real-time detection of amplicons or multiplexing the reactions, could further decrease the turnaround time, labor, and cost of tests. Although the PCR procedures described here are identical for the nine sets of primers, attempts to combine these together reproducibly in multiplex formats was unsuccessful (results not given). Multiplex PCR procedures for SEs have subsequently been described (2, 14), and the studies described here will shortly be extended to facilitate more rapid testing of cultures utilizing a similar approach.

A small number (4%) of discrepancies between the results with the PCR and the RPLA assays were obtained, and similar results have been reported elsewhere (2, 7, 14). Further testing of cultures giving discrepant results by ELISA gave similar (although not identical) results to the RPLA but not to the PCR. It is intriguing that these differences were both reproducible and strain specific, and further investigation, for example by the analysis of DNA sequence, may reveal additional differences within the SE genes.

It was noted previously that strains of S. aureus producing SEA–E in vitro often reacted with phages in group III of the international set (including I and III) and similarly production of TSST-1 is associated with phage-group I (20). Results presented here for the detection of the genes of the same toxins are consistent with these observations. The associations previously reported between SEB and phage group V and between SEC and SED and phage 95 (20), however, were not detected here (results not shown). Strains where SEG, SEH, and SEI gene fragments were detected similarly often reacted with phages within group III. It may be of note that cultures of S. aureus where the exotoxin genes were not detected often showed different or no phage susceptibilities to those containing SE genes and hence are likely to represent a different major group within this bacterial species.

A previous analysis of S. aureus strains collected between 1969 and 1990 using methods to detect SEA–E, showed that SEA, SEA/SED, and SEC/SED were responsible for 57%, 15%, and 8%, respectively, of cases of SFP in the United Kingdom (20). In this study (where >90% of the cultures were isolated after 1990) SEA, SEG, SEH, and SEI were detected most frequently and were found (together with other toxins) in 23%, 44%, 21%, and 21% of the incidents of suspected SFP. Although there was bias in the selection of some of the cultures for this study to include strains where enterotoxins had previously not been detected, these results suggest that the role of all SEs in human disease should be re-evaluated. Cultures collected over the past 30 years in the FSML will shortly be re-examined on a more systematic basis to establish if, as suggested by the results presented here, there has been a change in the contribution of the different enterotoxins involved with incidents of SFP.

It was previously suggested that SEs other than SEA–D may be involved with human disease in the United Kingdom (20). Two cultures from a United Kingdom incident of SFP in 1969 previously identified as emetic by monkey-feeding tests (M. S. Bergdoll, University of Wisconsin, Madison, Wis.) but that did not produce SEA–D in vitro (20) were both shown in this study to contain the SEH gene fragment here. No enterotoxin genes were detected in a further culture (also from a possible SFP incident in 1969) with identical emetic properties. A further SE has been described (23) designated SEJ. The cultures described here will shortly be investigated for the presence of the gene for this toxin, particularly those from suspected SFP where no other enterotoxins have previously been detected.

Problems with the application of PCR-based procedures directly to foods are well recognized (21). Failures of PCR to amplify specific target DNA in extracts from foods could be due to an inability to extract sufficient intact target DNA or to the copurification of inhibitors to the reactions. A simple aqueous extract of food sample combined with further dilutions in water is described here, and no attempts were made to purify further the DNA prior to analysis by PCR. Although this method was clearly not suitable for the detection of SEs in the dairy products, the sensitivity of the PCR when applied to other food types (mushroom soup and ham) was similar to that of the control samples. The method was also successful in the detection of a range of SEs following multiplication of S. aureus in food matrices, including those that were naturally contaminated by this bacterium. It may be of note that the numbers of organisms required to amplify DNA using this procedure is similar to the high numbers of S. aureus required for the production of sufficient enterotoxin in foods to produce SFP, which is on the order of 10^5 CFU/g (20). A simple DNA extraction method is currently being evaluated that may make this method more applicable to analysis of a wider range of foods.

Currently the detection of SEs directly in foods is labor intensive, limited to only certain SEs where reagents are available, and is relatively insensitive (17). The protocols described here have demonstrated the appropriate target DNA in naturally contaminated foods (even in one sample after 15 years storage at room temperature) and, together with further DNA purification methods, may offer suitable alternatives for the detection of enterotoxin in a wide range of food types.

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REFERENCES

1. Barrow, G. I., and R. K. A. Feltham. 1993. Cowan and Steel's manual for identification of medical bacteria, 3rd ed. Cambridge University Press, Cambridge.

2. Becker, K., R. Roth. and G. Peters. 1998. Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin 1 gene. J. Clin. Microbiol. 36:2548–2553.

3. Bergdoll, M. S., B. A. Crass, R. F. Reiser, R. N. Robbins, and J. P. Davis. 1981. A new staphylococcal enterotoxin, enterotoxin F, associated with the toxic shock syndrome *Staphylococcus aureus* isolates. Lancet i:1017–1021.

4. Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim van Dillen, and J. van der Noordaa. 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. 28:495–503.

5. De Saxe, M., A. W. Coe, and A. A. Wieneke. 1982. The use of phage-typing in the investigation of food poisoning caused by *Staphylococcus aureus* enterotoxins, p. 173–197. *In* J. E. L. Corry, D. Roberts, and F. A. Skinner (ed.), Isolation and identification methods for food poisoning organisms. Society for Applied Bacteriology Technical Series No. 17. Academic Press, London.

6. Jablonski, L. M., and G. A. Bohach. 1997. *Staphylococcus aureus,* p. 353–375. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.

7. Johnson, W. M. S., S. D. Tyler, E. P. Ewan, F. E. Ashton, D. R. Pollard, and K. R. Rozee. 1991. Detection of genes for enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by polymerase chain reaction. J. Clin. Microbiol. 29:426–430.

8. Lachica, R. V. F., C. Genigeorgis, and P. D. Hoeprich. 1971. Metachromic agar-diffusion method for detecting staphylococcal nuclease activity. Appl. Microbiol. 21:585–587.

9. Marples, R. R., and A. A. Wieneke. 1993. Enterotoxins and toxic shock syndrome toxin-1 in non-enteric staphylococcal disease. Epidemiol. Infect. 110:477–488.

10. Morris, C. A., H. D. Conway, and P. H. Everall. 1972. Food-poisoning due to staphylococcal enterotoxin E. Lancet ii:1375–1376.

11. Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. Infect. Immun. 66:3337–3348.

12. Ren, K., J. D. Bannan, V. Pancholi, A. L. Cheung, J. C. Robbins, V. A. Fischetti, and J. B. Zabriske. 1994. Characterisation and biological properties of a new staphylococcal exotoxin. J. Exp. Med. 180:1675–1683.

13. Roberts, D., W. Hooper, and M. Greenwood. 1995. Practical food microbiology, 2nd ed. Public Health Laboratory Service, London.

14. Schmitz, F. J., M. Steiert, B. Hofmann, J. Verhoef, U. Hadding, H. P. Heinz, and K. Köhrer. 1998. Development of a multiplex-PCR for direct detection of the genes for enterotoxin B and C, and toxic shock syndrome toxin-1 in *Staphylococcus aureus* isolates. J. Med. Microbiol. 47:335–340.

15. Simkovicova, M., and R. J. Gilbert. 1971. Serological detection of enterotoxin from food poisoning strains of *Staphylococcus aureus*. J. Med. Microbiol. 4:19–30.

16. Su, Y. C., and A. C. L. Wong. 1995. Identification and purification of a new staphylococcal enterotoxin. H. Appl. Environ. Microbiol. 61:1438–1443.

17. Su, Y. C., and A. C. L. Wong. 1997. Current perspectives on detection of staphylococcal enterotoxins. J. Food Prot. 60:195–202.

18. Wieneke, A. A. 1988. The detection of enterotoxin and toxic shock syndrome toxin-1 production by strains of *Staphylococcus aureus* with commercial RPLA kits. Int. J. Food Microbiol. 7:25–30.

19. Wieneke, A. A. 1991. Comparison of four kits for the detection of staphylococcal enterotoxin in foods from outbreaks of food poisoning. Int. J. Food Microbiol. 14:305–312.

20. Wieneke, A. A., D. Roberts, and R. J. Gilbert. 1993. Staphylococcal food poisoning in the United Kingdom, 1969–90. Epidemiol. Infect. 110:519–531.

21. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63:3741–3751.

22. Woolaway, M. C., C. L. R. Bartlett, A. A. Wieneke, R. J. Gilbert, H. C. Murrell, and P. Aureli. 1986. International outbreak of staphylococcal food poisoning caused by contaminated lasagne. J. Hyg. 96:67–73.

23. Zhang, S., J. J. Iandolo, and G. C. Stewart. 1998. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (sej). FEMS Microbiol. Lett. 15:227–233.

EXHIBIT B

2. Staphylococcal Enterotoxin (SE)

SE is a simple protein having a molecular weight of approximately 27,000 and containing a single -S-S- bond within the molecule. According to the difference of antigenicity, it is classified into 5 species of A to E. C type is further classified into 3 types of C1, C2 and C3 according to the difference of isoelectric point. However, they are immunologically identical. It is presumed that amino acid sequence thereof adjacent to the -S-S- bond in the molecule resemble closely although the antigenicity is different, and the amino acid sequence relates to emetic activity of SE.

Recently, SE F, G, H, I, J, K and L types are added other than A to E types. F type was named toxic shock syndrome toxin (TSST-1), which has been demonstrated to have no -S-S- bond within the molecule and to be a toxin having different structure from SE. Currently reported SE are all listed in Table 1. SE I, K and L types comprise only 1 molecule of cysteine within the molecule, and no -S-S- bond. Further, SE J, K and L types have been demonstrated for super antigen activity, its emetic action has not been studied yet, however.

On the other hand, it has been proved that SE, TSST-1, streptococcus pyrogenic toxin or the like produced by staphylococcus aureus have action of specifically activating T cell and named as "bacterial super antigen". Therefore, the super antigen activity which is a mitogenic activity of T cell and can be studied easier than the emetic action is now being researched. Hereafter, when a research for structural gene of staphylococcus aureus proceeds, there is a possibility for a further new SE type is added. However, it is considered that the bacterial super antigen activity has no direct relation to the emetic activity. There is also a possibility that while the emetic action remains

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unexamined, all of bacteria having the super antigen activity will be named as SE. If such situation continues, two species of SEs, namely the one causing staphylococcal food poisoning and the one containing only the super antigen activity will be coexisted, and possibly lead to great disorder.

Table 1

Physicochemical and genetic property of staphylococcal enterotoxin

Enterotoxin type	Molecular weight	Amino acid residue	Isoelectric point (pI)	N terminus amino acid	Structural gene
A	27078	233 + 24	7.3	Ser	C or B
B	28336	239 + 27	8.6	Glu	C or P
C1	27946	239 + 27	8.6	Glu	C or P
C2	27589	239 + 27	7.0	Glu	C
C3	27563	239 + 27	8.1	Glu	C
D	26360	228 + 30	7.4	Ser	P
E	26425	230 + 27	7.0	Ser	B (?)
(F	22049	194 + 40	7.2	Ser	C : TSST-1)
G	27043	233 + 25	?	Gln	?
H	25145	194 + 24	5.65	Glu	?
I	24928	217 + 24	?	Gln	?
J	?	269	?	?	?
K	25539	219 + 23	6.5	Gln	?
L	?	?	?	?	?

B: Bacteriophage ; C: Chromosome ; P: Plasmid ; ?: Unidentified

(END)

<情報>
ブドウ球菌エンテロトキシン

1. はじめに

2000年6月末〜7月上旬に雪印乳業（株）の加工乳や乳飲料を原因とする患者数13,420名にも及ぶ戦後類をみない大規模なブドウ球菌食中毒が発生した。この事例は原因食品からはブドウ球菌エンテロトキシン（以下SEと略）Aのみが検出され、黄色ブドウ球菌が検出されない典型的な毒素型食中毒であった。この事例はこれまでのブドウ球菌食中毒ではあまり問題にされてこなかった検査法の検出限界や検査法の精度、乳製品からのSEの検出法、加熱殺菌後の検体からのSEの検出、SEの発症量など、様々な課題を提起した。この機会に近年のSE研究の概要を記述する。

2. ブドウ球菌エンテロトキシン（SE）

SEは分子量27,000前後の単純蛋白質で、分子内に1個の-S-S-結合を有している。抗原性の違いによりA〜Eの5種類に分けられている。C型は等電点の違いによりC1, C2, C3の3型に分けられているが、免疫学的には同一である。抗原性が異なっていても、分子内-S-S-結合の近傍のアミノ酸配列が酷似しており、そのアミノ酸配列がSEの催吐活性と関わっていると推測されている。

近年、SEA型〜E型以外にF型、G型、H型、I型、J型、K型、L型が追加されている。F型はトキシックショック症候群毒素（TSST-1）と命名され、分子内に-S-S-結合がなく、SEとは構造が異なる毒素であることが明らかになっている。表1は現在報告されているSEをまとめたものである。SEI型、K型、L型には分子内にシステイン1分子のみが存在し、分子内に-S-S-結合はない。また、SEJ型、K型、L型はスーパー抗原活性について証明されているが、催吐作用については検討されていない。

一方、黄色ブドウ球菌が産生するSEやTSST-1、レンサ球菌発熱性毒素などはT細胞を特異的に活性化する作用があることが証明され、「細菌性スーパー抗原」と呼称されている。そのため、催吐作用よりも簡単に調べられるT細胞のマイトジェン活性であるスーパー抗原活性が調べられている。今後、黄色ブドウ球菌の構造遺伝子の研究が進めば、さらに新しいSE型が追加される可能性がある。しかし、細菌性スーパー抗原活性と催吐活性は直接関係がないと考えられている。催吐作用が未検討のまま、スーパー抗原活性を有するものすべてが、SEという名称で呼ばれる可能性がある。このようなことが続けば、ブドウ球菌食中毒を起こすSEとスーパー抗原活性のみを有するSEの2種類が存在し、大きな混乱を生ずることになる。

3. 検査法

SE検査にはSEに対する抗体を用いる血清学的な手法が利用されている。SEの研究は毒素を高純度に精製し、ウサギを免疫して特異性の高い抗血清を作製し、それを用いて毒素を検出する方法の開発から始まった。研究の当初は種々のゲル内沈降反応、逆受身赤血球凝集反応、ラジオイムノアッセイ、酵素抗体法、逆受身ラテックス凝集反応等が開発された。その結果、現在のような高検出感度のキットが市販されるようになった。

わが国で入手可能なキットの種類を表2に示した。表に示したSE検査キットの検出感度は各社でそれぞれ表示されており、0.2〜2ng/mlである。しかし、どのメーカーのキットであっても検出限界に近い濃度の毒素量を正確に検出することは困難である。SEを検出するための所要時間は各社のキットで異なる。

食品からのSE検出は各社キット添付の説明書に従って試料を調製して実施する。にぎりめしや弁当などのブドウ球菌食中毒事件では食品1g中に0.2〜1.28μgのSEが検出される場合が多い。このような検査では現在市販されている検査キットの検出感度で十分であった。しかし、雪印ブドウ球菌食中毒事件の場合は、検査材料が加工乳や乳飲料で、黄色ブドウ球菌が検出されず、さらに含有するSE濃度が市販検査キットの検出感度以下であった。そのため試料の濃縮やその後の除

表1 ブドウ球菌エンテロトキシンの物理化学的および遺伝学的性状

エンテロトキシン型	分子量	アミノ酸残基数	等電点(pI)	N末端アミノ酸	構造遺伝子
A	27078	233 + 24	7.3	Ser	C or B
B	28336	239 + 27	8.6	Glu	C or P
C1	27946	239 + 27	8.6	Glu	C or P
C2	27589	239 + 27	7.0	Glu	C
C3	27563	239 + 27	8.1	Glu	C
D	26360	228 + 30	7.4	Ser	P
E	26425	230 + 27	7.0	Ser	B (?)
(F	22049	194 + 40	7.2	Ser	C　　:TSST-1)
G	27043	233 + 25	?	Gln	?
H	25145	194 + 24	5.65	Glu	?
I	24928	217 + 24	?	Gln	?
J	?	269	?	?	?
K	25539	219 + 23	6.5	Gln	?
L	?	?	?	?	?

B: バクテリオファージ；　C: クロモゾーム；　P: プラスミド；　?: 不明

表2　市販ブドウ球菌エンテロトキシンキット

商品名	取り扱い会社	検出感度	所要時間
SET-RPLA「生研」	デンカ生研（株）	1-2ng/ml	18-20時間
バイダスSET	日本ビオメリュー（株）	0.25-1ng/ml	80分
黄色ブドウ球菌毒素 ELISA kit SETVIA48 (TECRA製)	セティカンパニー リミテッド	1ng/ml	約4時間
黄色ブドウ球菌毒素 ELISA kit R4101 (R-Biopharm製)	同上	1ng/ml	約4時間
トランジアプレートブドウ球菌エンテロトキシン (Diffchamb製)	メルク・ジャパン（株）	0.25-1ng/g	約90分
RIDAスクリーン黄色ブドウ球菌エンテロトキシン	アツマックス（株）	0.2-0.7ng/ml	約3.5時間